STUDIES FOR STERILIZATION OF SPACE PROBE COMPONENTS

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TABLE OF CONTENTS

I. ABSTRACT

The research presented in this final report for NASw-550 constitutes the results of an extension of the work to study methods of sterilization for space probe components by the Wilmot Castle Company Laboratories initiated under a preceding contract (NASr-31). Emphasis has been placed on the investigation of dry heat sterilization processes in the temperature range of 80°C to 160°C, particularly at temperatures of 125°C and 135°C.

In the studies reported here it has been attempted to further define the various biological, chemical, and physical factors that can influence the effectiveness of dry heat as a sterilizing process. The areas of investigation include:

1 - The establishment of the nature of the killing effect of dry heat on organisms in soil which are resistant to dry heat.

2 - The effects of combinations of dry heat and gamma irradiation treatment on bacterial spores.

3 - The effects of entrapment of organisms in non-aqueous liquid (oil).

4 - A comparison of common bacteriological media as to their ability to recover organisms which have been exposed to dry heat treatments.

5 - The determination of the time required to effect the sterilization of microorganisms in soil from 80°C through 160°C.

A summary of the findings to date is as follows:

1 - Survivor curves have been obtained for both pure cultures of bacterial spores in soil and the indigenous microbial flora of soils. These curves have definite tailing characteristics as compared to such curves for pure cultures of microorganisms on other less protective carriers (e.g. paper strips).

2 - Certain combinations of gamma irradiation with dry heat treatments have been found to kill dry spores of Bacillus subtilis var. niger and Bacillus coagulans more readily and with less severe doses than with either agent alone. An increased but not truly additive killing effect was observed when both agents were employed simultaneously.

3 - Bacterial spores of Bacillus subtilis var. niger entrapped or treated in a non-aqueous medium such as mineral oil, are apparently no more resistant than when treated in air.

4 - Common bacteriological culture media have been found to differ in their ability to recover heat-injured microorganisms. Tryptone glucose yeast extract broth yielded recoveries at much longer heating times than

trypticase soy broth, thioglycollate broth (a common sterility test medium), and a soil extract broth.

5 - A linear thermal death time curve has been obtained for the mesophilic aerobic flora of microorganisms in garden soil in the temperature range of 80°C through 160°C.

II INTRODUCTION AND REVIEW OF THE CURRENT LITERATURE

The references pointing out the need for, and the plausible modes of, sterilizing interplanetary unmanned spacecraft reported by the scientific community at large were cited previously (5). The preferred technique should be dry heat in the final sealed container, with no access permitted nor mechanically possible thereafter, except with complete re-sterilization.

NASA not only has established a more definitive policy and responsibility but has undertaken additional necessary research to advance the state-of-the-art with procedures and measures for attaining this requirement (3,8).

The research undertaken under this contract has been specifically oriented toward obtaining basic information on the resistance of microorganisms so as to better define the dry heat cycles which would be required to sterilize spacecraft.

At the time of the inception of the work on this contract, it was presumed that the spacecraft would be assembled and then sterilized as a complete assembly. In developing the data which was necessary to establish a dry heat sterilization cycle with a specific probability of not having a single micro-organism survive, a search was initiated to determine the types of contaminants and their resistance to the presumptive

situation. It had been clearly established in earlier work (5,6) that two of the more resistant conditions, as far as resistance of microorganisms to dry heat is concerned, was that of soil contamination with its indigenous microbial flora, and the entrapment or existence of microorganisms or microbial spores in the interior of materials and/or electronic components. Therefore, if the nature of the dry heat cycles required to render materials containing such contaminants sterile were determined, adequate treatment cycles could be established with the desired degree of reliability. For this reason much of the research has been on the aspect of determining the dry heat cycles necessary to sterilize soil samples.

Recently, many views and partial solutions on the above problem have been advanced. The one of major concern has been the fact that spacecraft and/or components can be and are now being manufactured and assembled under cleaner conditions through the employment of "clean room" techniques. This of course assumes that much of the biological contamination can be controlled; in fact, controlled to the point where no soil would be deposited on or incorporated into the spacecraft. Therefore, a re-evaluation of the ultimate dry heat cycle to render a spacecraft sterile is required. Future work will be concerned with evaluating some of the actual conditions which exist, and that should be taken into account in designing

adequate sterilization cycles.

In addition to the work cited previously, several of the practical studies which have come to our attention are those by the Fort Detrick (9,10) and the Air Force groups (2,7). The Fort Detrick group has studied and reported on a) levels and resistance of the microbial contamination obtained on surfaces exposed to room air or touched by the human hand (9), and b) the dry heat cycles which would sterilize naturally contaminated metal surfaces (10). In their study on the level of microbial contamination, the results indicate that the maximum viable microbial contamination expected in practice on a space craft is probably a few orders of magnitude lower than Hobby's previous estimate of 10^9 organisms on the surface and interior of a spacecraft (4). In evaluating the dry heat cycle required to sterilize naturally contaminated materials, the Detrick group found that the samples exposed to 135°C for 12 hours contained no viable microorganisms.

The Air Force contracted and supported "in-house" and extramural studies to develop sterilization procedures for deliberately contaminated electronic components (2,7). The results of treating such components contaminated with spores of Bacillus subtilis var. niger or Bacillus stearothermophilus indicated that dry heat cycles longer than 12 hours at 125°C

were required for sterilization. However, spores of these organisms were not recovered from any of the components that were examined after 24 hours at 125°C (2). The levels of spores were 10^5-10^6 per component.

In contrast to the adequacy of these comparatively short cycles, the results of our earlier investigation indicate that a much longer time is required if soil is present on or in the spacecraft. Bruch (1) has reviewed this aspect and points out that some new rationale for the dry heat cycles would be required in order to yeild internally sterile but useable space probes. He further suggests a more realistic basis for judging the adequacy of such treatments.

This report presents data corroborating our earlier findings on the resistance of organisms in soil. Results of an investigation of the effects of irradiation treatment in conjunction with dry heat on bacterial spores is also presented. The resistance of organisms in or under oil, and some indications of the ability of several common culture media to recover heat injured spores was also studied.

III STUDIES ON THE RESISTANCE OF DRY MICROBIAL SPORES IN SOIL TO DRY HEAT.

From the literature and the data presented in previous work on this subject, it is apparent that clean pure cultures of some dried spores do follow such an exponential pattern after an initial period. Due to the high degree of resistance noted for a part of the microbial flora in dry soil samples, and on other artificial carriers, it was desirable to determine the pattern of destruction of such spores by obtaining survivor curves.

Among the specific studies which have been completed and are reported in this section are the survivor curve studies a) on the natural microbial population of dry soil samples with particular reference to the mesophilic aerobic sporeforming group of microorganisms, and b) on sterile dry soil samples to which spores of several heat resistant isolates have been added.

Samples (0.1 g per test tube) of dry garden soil (FG) were treated at various temperatures from 80°C to 160°C in the previously described dry heat units (5) for various times. Multiple samples were then assayed quantitatively, employing conventional plate count procedures for the level of surviving organisms. It had been found that a 10 minute ultrasonic treatment of the original dilution dispersed the organisms

better than shaking. Multiple experiments were run, and each sample was plated in triplicate for each dilution. The results obtained were plotted on semi-log paper and are presented in Figure 1.

The type of curves obtained indicate a non-logarithmic death rate, however, each curve could represent a composite of curves which would be obtained from treating a heterogeneous population of organisms with their resistance to dry heat. This heterogeneity could be due to a) either the variation in the inherent heat resistance of the population of organisms,or b) the alteration of the heat resistance of some members of the population due to physical and/or chemical protection by the menstruum.

In either case, the observed survivor curve drawn at any one temperature would be a composite of two or more survivor curves reflecting the variation in heat resistance of each member of the heterogeneous group. The death rate for each member could be logarithmic and hence give resultant curves as observed in Figure 1.

If the survivor curves of best fit are projected to the base line, the times extrapolated for reduction to one organism or less (no detectable survivors) do agree closely with the values obtained by endpoint or partial survival data studies for the respective temperatures. The basis for comparing

these sets of data is that, although one recovery medium was a solid (agar base) and the other a liquid (broth), both were compounded of the same nutritional ingredients and all other techniques of handling were identical.

Several survivor curves were also obtained for a) pure cultures of Bacillus subtilis var. niger and Bacillus coagulans spores in sterilized soil, and b) for two pure cultures of heat-resistant isolates added back to sterilized soil (these organisms are referred to as soil isolates 69C and 541, respectively). The curves obtained are presented in Figures 2, 3, 4 and 5, respectively. The thermal death time and times required to sterilize equivalent samples of these spore-soil samples as determined by partial survival technique are given in Table 1. These curves are not unlike those obtained for soil samples with their indigenous microflora and their heterogenous resistance. By comparing the shape of the curves found for the organisms on paper strips versus the shape obtained for inoculated soil, the degree of enhancement of resistance is apparent.

IV COMPARISON OF MEDIA FOR RECOVERY OF HEAT INJURED SPORES

The specific studies which have been completed, and are reported in this section, are the partial survival or end-point studies on the same soil samples reported in III above. These studies were extended so as to compare the ability of various nutrient media to recover low numbers of residual surviving organisms from heat treated samples. The results might also provide a direct comparison to the data obtained by the survivor curves technique.

Since the inception of this work, the recovery media of choice have been the generally accepted standard sterility test medium, thioglycollate broth, and trypticase soy broth, the latter having been found empirically to afford better recoveries for several of the test organisms. No panoramic comparison of media or specific additives was attempted. The media employed in this work included thioglycollate broth[1], trypticase soy broth[1], tryptone glucose yeast extract broth[2], and a soil extract broth.[2]

The results obtained so far in the course of this work are reported in Table 2 and Figure 8. Tryptone glucose yeast extract broth was the one medium which recovered aerobic mesophilic organisms from samples of soil treated with dry heat when any of the other media failed to give evidence of

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1 Baltimore Biological Laboratories, Inc., Baltimore 18, Maryland
2 See Appendix A for specific ingredients

growth.

It would appear redundant, but valuable, to employ several media for sterility testing of treated materials, until such factors which provide a heat injured organism the best chance of surviving and growing are all known.

V STUDIES ON THE RESISTANCE OF MICROORGANISMS IN SOIL TO DRY HEAT AT 80°C AND 100°C

Studies to determine the resistance of organisms in soil have been underway at temperatures of 80°C and 100°C. The results are given in Table 3 for 0.1 g samples of soils FG (fresh garden) and CO (soil from cow pasture). When the thermal death time values obtained with thioglycollate broth are plotted on a semi-log scale a linear curve can be drawn from 80°C to 160°C. A similar relationship was found for thermal death time values obtained with tryptone glucose yeast extract broth through 120-160°C; the data at 80°C and 100°C is not available at this time.

Figure 8 presents a summary of the resistance of organisms in soil FG to dry heat in the temperature range 80°C to 160°C, employing two different media for recovery broths.

VI STUDIES ON THE EFFECTS OF COMBINATIONS OF DRY HEAT AND GAMMA IRRADIATION ON BACTERIAL SPORES

A brief literature survey was made to evaluate the feasibility of employing sub-sterilizing, but lethal, combinations of dry heat and gamma irradiation as a mode of sterilization. The information readily available indicated that these agents can be effective in combination under certain conditions, and that their combination may be more than additive with regards to their killing effect on microorganisms. The results given in the literature are not well quantitated, particularly with regard to the effects on dry bacterial spores.

In order to determine if any selected combination of the two agents were effective and to what degree, studies were performed by exposing two species of bacterial spores, dried on filter paper strips, to dry heat treatment at 120°C prior to, simultaneous with, and after gamma irradiation at 2×10^4 rad per hour.

Preliminary results (5) indicated an increased sporicidal effect on dry spores of Bacillus subtilis var. niger and Bacillus coagulans when both agents were employed in certain combinations as compared to either agent independently.

Additional experiments were undertaken and the results of the combined treatments as well as the respective effects of each agent independently on dry spores of Bacillus subtilis var. niger are presented in Figure 7, and on dry spores of

Bacillus coagulans, in Figure 8.

It was necessary to devise a special unit to treat the spores with heat and irradiation simultaneously. The unit is diagrammatically described in the previous progress report (6). The materials of construction were aluminum and plexiglas. The temperature was controlled at 120°C ± 1°C.

The results indicate that simultaneous treatment with dry heat and irradiation was one of the most effective combinations of those studied in destroying the spores of both organisms. Death occurred more rapidly than with any other combination of treatments and had an increased effect over either agent independently. Treatment with dry heat prior to irradiation was comparable in effectiveness to that obtained when both agents were applied simultaneously to spores of B. coagulans. Data on heat treatment after irradiation was not continued to a sterile end point. All other combinations of treatments were less effective on dry spores of both species of organisms, but more effective than either agent independently.

In evaluating the degree of increase of spore destruction, it was concluded that all combinations of treatments fell short of being truly additive. The results, even though treatments were carried out under a limited set of conditions, indicated that both agents could be employed to effectively sterilize by combining sub-sterilizing doses of each agent simultaneously or in sequence.

VII STUDY OF THE RESISTANCE OF BACTERIAL SPORES TO DRY HEAT WHEN TREATED IN A NON-AQUEOUS MEDIUM

In an attempt to evaluate the effect of dry heat on bacterial spores in a non-aqueous, non-aerobic menstruum, bacterial spore strips were treated in dry, previously sterilized mineral oil (extra heavy liquid petrolatum, USP) in screw capped test tubes. The tubes were treated in the cylindrical aluminum blocks at temperatures of 120, 140, and 160°C. Spores of Bacillus subtilis var. niger and Bacillus coagulans on paper strips at a level of 1 million per strip were employed. Initially the strips to be treated were placed in tubes of oil at room temperature; the tubes were then placed in the heat blocks, removed at various time intervals, and cooled. Such treatments included thermal lag times. The strips were then removed aseptically and placed in sterile trypticase soy broth. The culture tubes were agitated and incubated for at least two weeks. Sterile medium was also added to the treatment tubes containing the oil. Residual oil on the strips did not interfere with the outgrowth of surviving spores. Very few of the cultured tubes which contained the treatment oil ever showed evidence of growth due to spores being washed from the paper by the oil, indicating that no significant loss of spores from the strips occurred. Quantitative controls presented no indication of any sporicidal effect by the oil.

The results of this series of tests are given in Table 4. They indicate that dry spores (on paper strips) under or in a dry non-aqueous menstruum were no more difficult to kill than they were in air. This was true at the temperature of 120 and 140°C. The times, at 160°C, required to effect complete kills were longer than those times required in air, because they were uncorrected for lag time of warm-up. In the studies employing tubes of oil pre-heated to 160°C, it took much shorter times to achieve complete kills; in fact these showed that the time required to sterilize them was less than the time required to heat the quantity of oil to 160°C.

The results of these studies supplement the conclusions found previously for dry heat treatment of dry spores on paper strips where the air was removed by vacuum or replaced with an inert gas (5).

VIII DISCUSSION

Many of the basic concepts regarding sterilization, thermal resistance of microorganisms, dry heat as a mode of sterilization, and such areas as logarithmic death rate, etc., have been discussed in the final report of the previous contract (5).

It was a major objective in this investigation to ascertain if organisms in soil did follow a logarithmic death rate pattern when lethal dry heat treatments were imposed upon them. It can be concluded, from the shape of the survivor curves obtained from soil samples, that the total composite microbial flora did not die off exponentially, and that a certain low number of them were quite resistant and persisted for long times. The survivor curves obtained for pure cultures of organisms added back to sterile soil did indeed follow a similar pattern, inferring that the organisms acquired some degree of protection from the soil as a menstruum. On other carriers these same organisms, after an initial period, proceeded to die off exponentially. If one extrapolates the obtained curves to their points of sterility (less than one organism remaining viable) these do agree with the points obtained employing the partial survival or end-point technique.

How much one can extrapolate below the level of unit time after which no growth is noted, and infer the probability

of one organism surviving is questionable due to the shape of the survivor curve. One certainly gets into treatment times which are extremely long and impractical, if not destructive to the objects which he desires to sterilize.

A major consideration which should be taken into account as a result of this study is that soil, and as much other dirt and debris, should be eliminated from the vicinity of any spacecraft and/or components. This could be accomplished by most stringent "clean room" techniques. The corollary to this would be to then base any sterilization cycles on the actual level and type of contaminants and their resistance.

Indeed, if the level of contamination can be reduced, the feasibility of achieving the desired probability of sterility is enhanced. In fact, the same degree of probability of sterility could be achieved employing less severe treatments, because less resistant organisms would have to be destroyed.

The feasibility of employing irradiation alone as a mode of sterilization has been referred to in other considerations of this overall program, but objections have been raised because the levels required would affect the materials and components envisioned. The study in this project suggests the possibility of employing gamma irradiation with dry heat treatment. Combinations of doses of each agent

much below those required to accomplish sterility are indicated. The possibility of employing this mode of sterilization would appear to be even more advantageous if it can be assumed that only a minimal deleterious effect would be evidenced on the material(s) to be so treated. Only actual tests on such materials would elaborate this issue.

IX FUTURE WORK

Since the completion of this contract, a new contract (NASw-879) extending the investigation on dry heat sterilization has been entered into.

The areas of investigation under this new contract are briefly outlined below:

1 - Conduct studies on the dry heat resistance of micro-organisms entrapped from air samples.

2 - Conduct studies on the dry heat resistance of resistant bacterial spore formers on or in various carriers, including carbon black, and kaolin, and possibly some of poor heat conductivity.

3 - Conduct dry heat sterilization studies on electronic components artificially inoculated with quantitative amounts of heat resistant spores in the temperature range of 100 - 135°C.

4 - Conduct a limited sterilization study on commercially available components in the temperature range of 100 - 135°C.

5 - Study techniques which might aid in recovering heat injured microorganisms after prolonged periods of time.

6 - Study and evaluate possible flash methods of sterilization.

X FIGURES AND TABLES

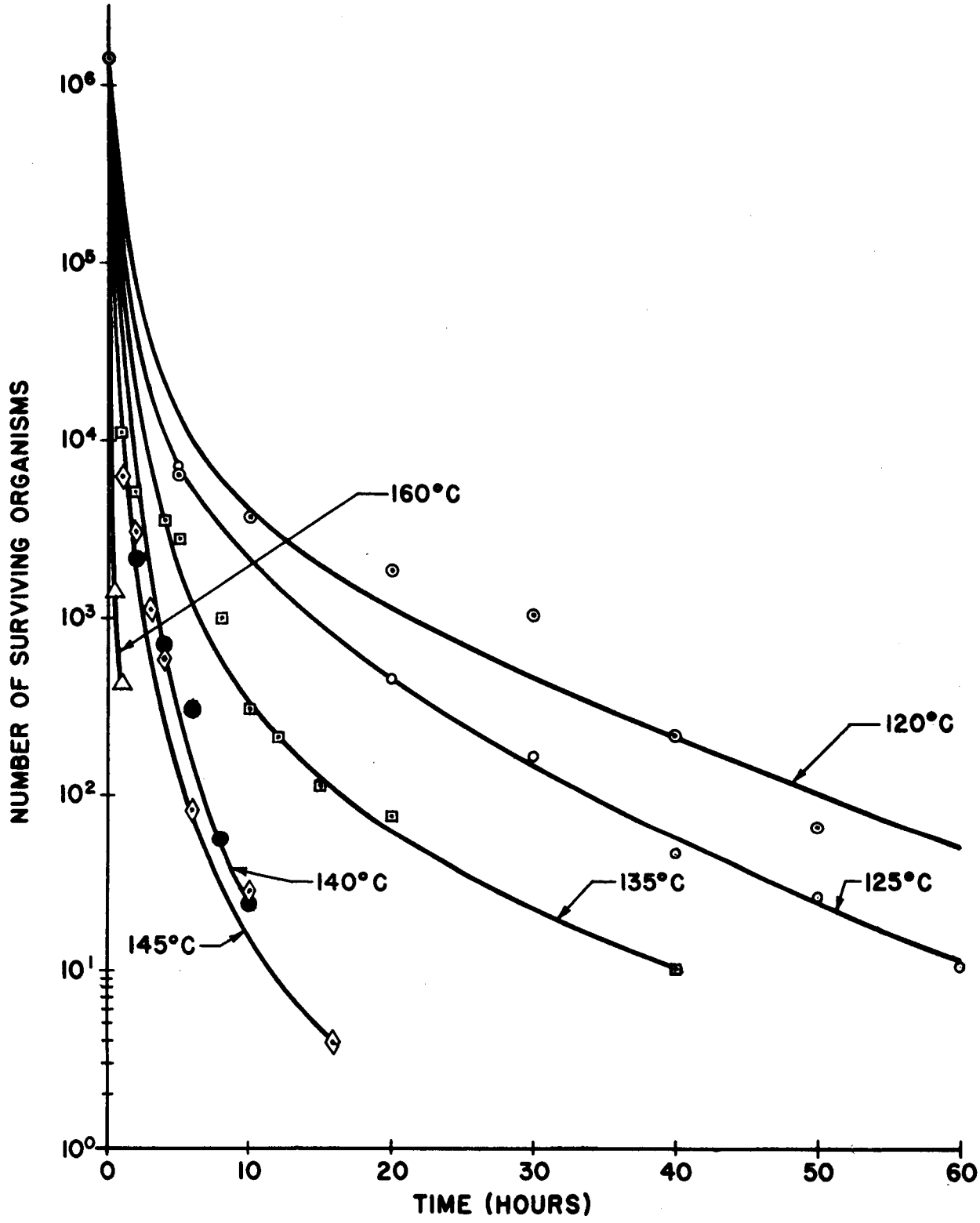

FIGURE 1. SURVIVOR CURVES OF ORGANISMS IN 0.1g SAMPLE OF DRY
FG SOIL TO DRY HEAT IN THE TEMPERATURE RANGE 120–160° C

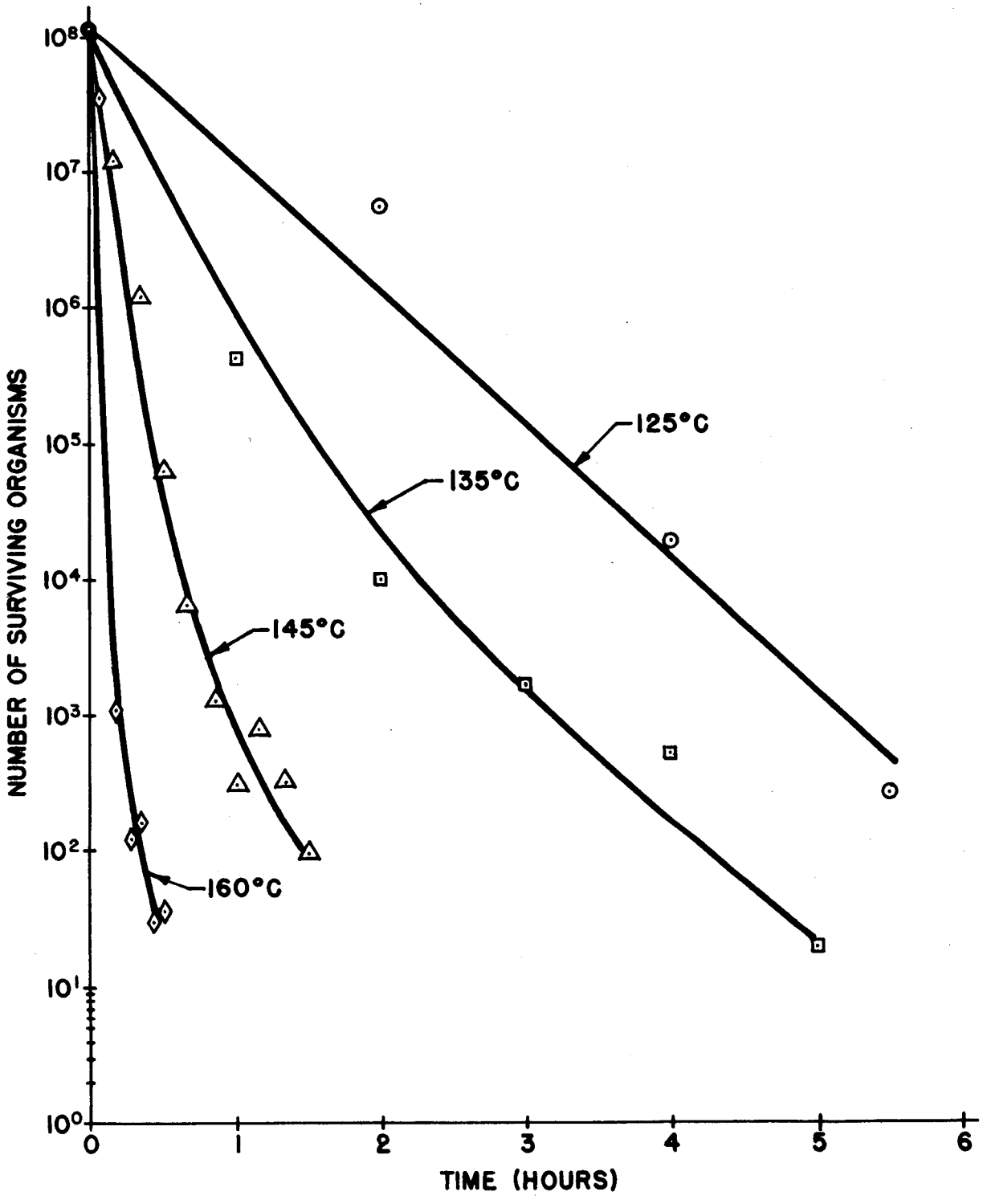

FIGURE 2. DRY HEAT SURVIVOR CURVES FOR 0.1g SAMPLES OF DRY FG SOIL INOCULATED WITH SPORES OF BACILLUS SUBTILIS VAR. NIGER

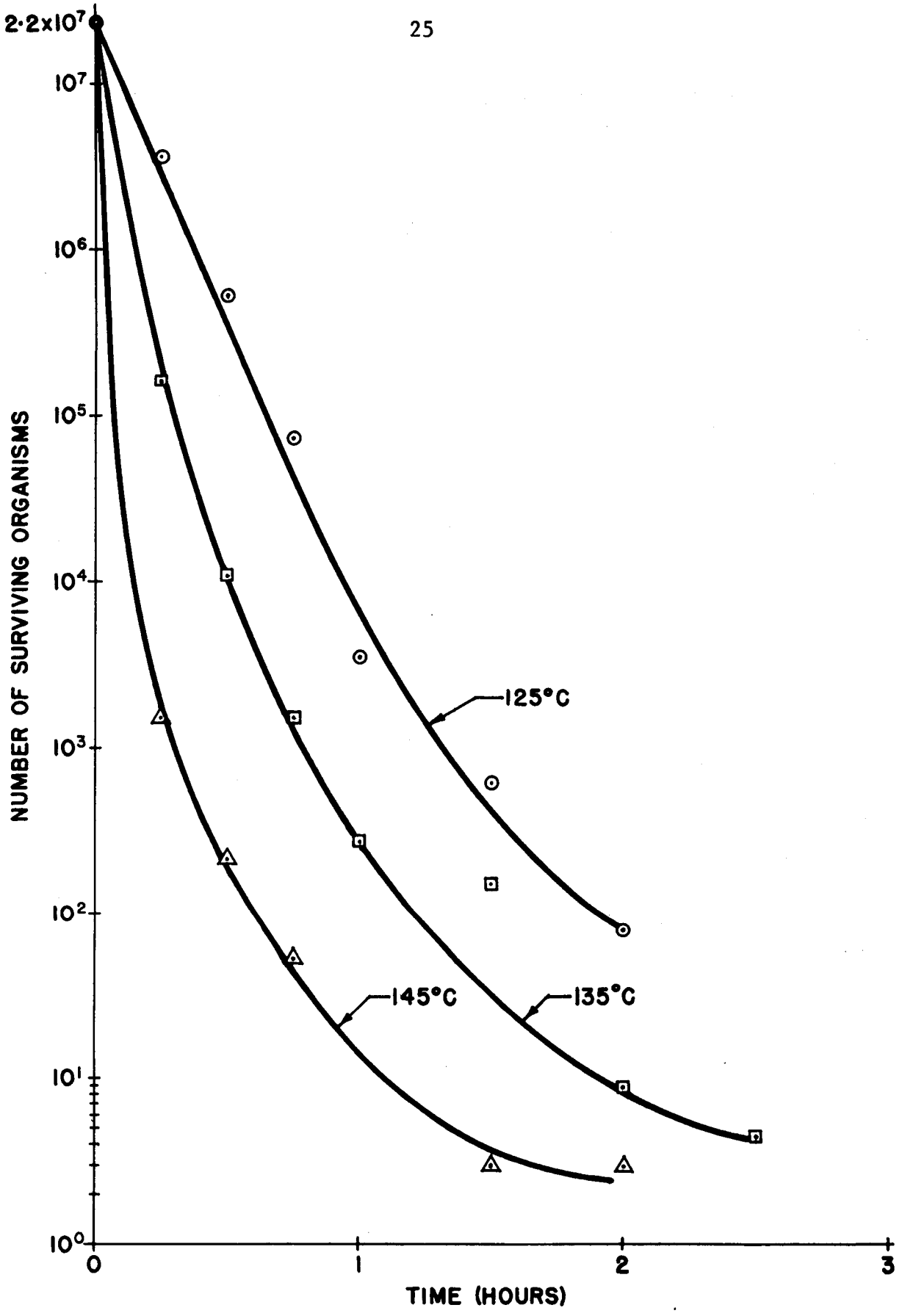

FIGURE 3. DRY HEAT SURVIVOR CURVES FOR 0.1g SAMPLE OF DRY FG SOIL INOCULATED WITH SPORES OF BACILLUS COAGULANS

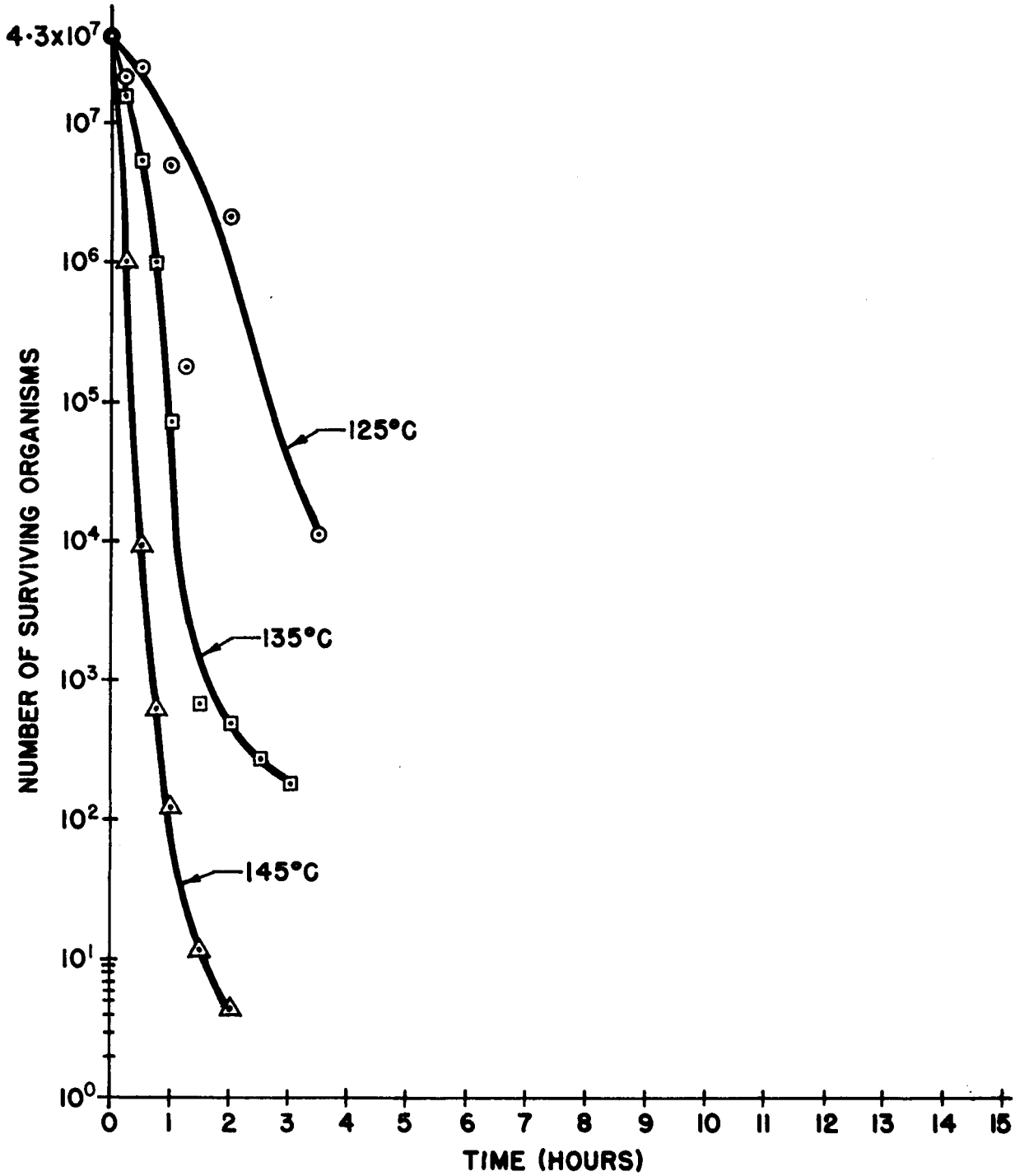

FIGURE 4. DRY HEAT SURVIVOR CURVES FOR 0.1g SAMPLE OF DRY FG SOIL INOCULATED WITH SPORES OF SOIL ISOLATE 69C

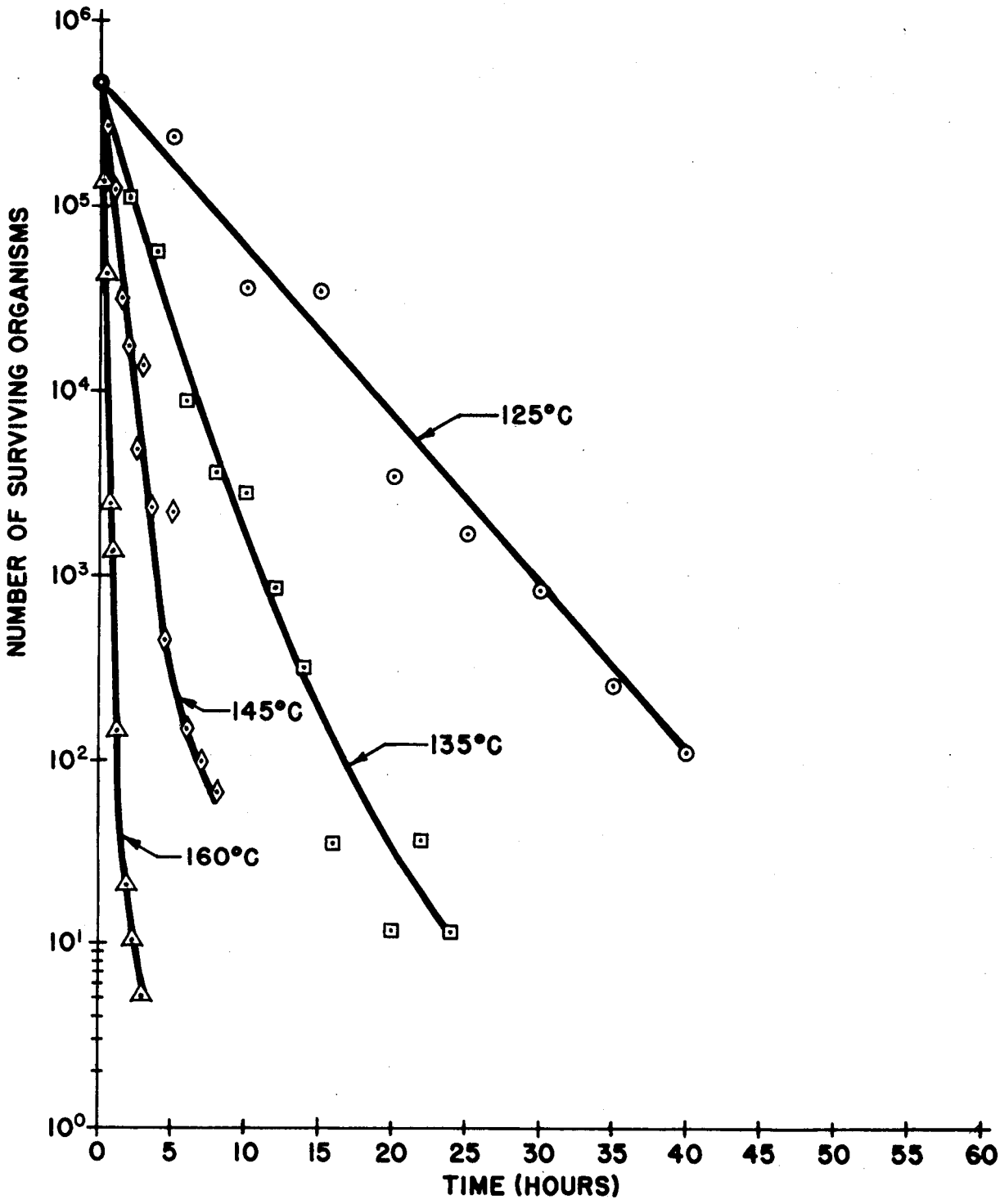

FIGURE 5. DRY HEAT SURVIVOR CURVES FOR 0.1g SAMPLE OF DRY FG SOIL INOCULATED WITH SPORES OF SOIL ISOLATE 541

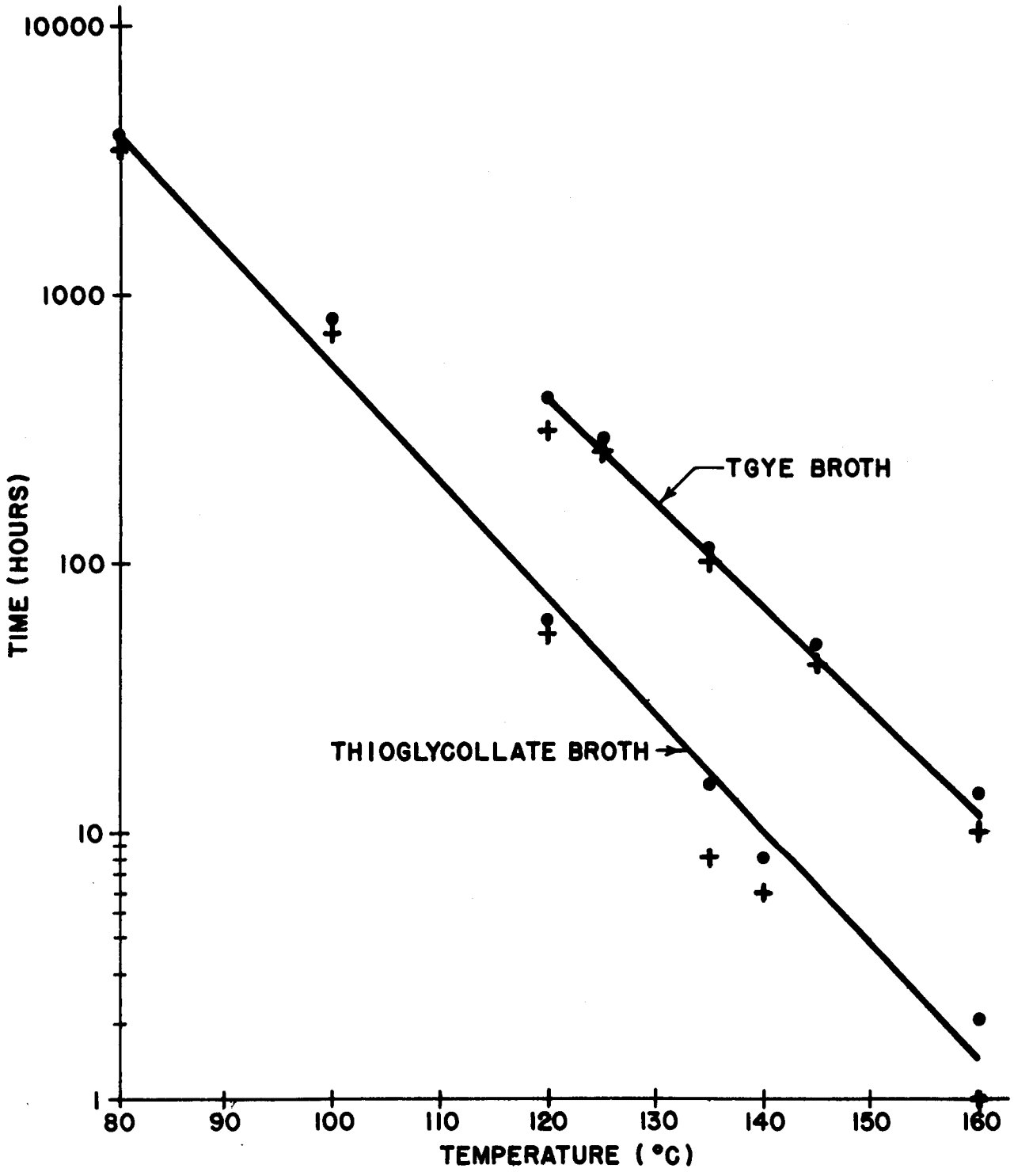

FIGURE 6. DRY HEAT THERMAL DEATH TIME CURVES FOR MESOPHILIC AEROBIC MICROBIAL POPULATIONS OF 0.1g SAMPLES OF DRY FG SOIL EMPLOYING TWO RECOVERY MEDIA IN THE TEMPERATURE RANGE 80–120°C.

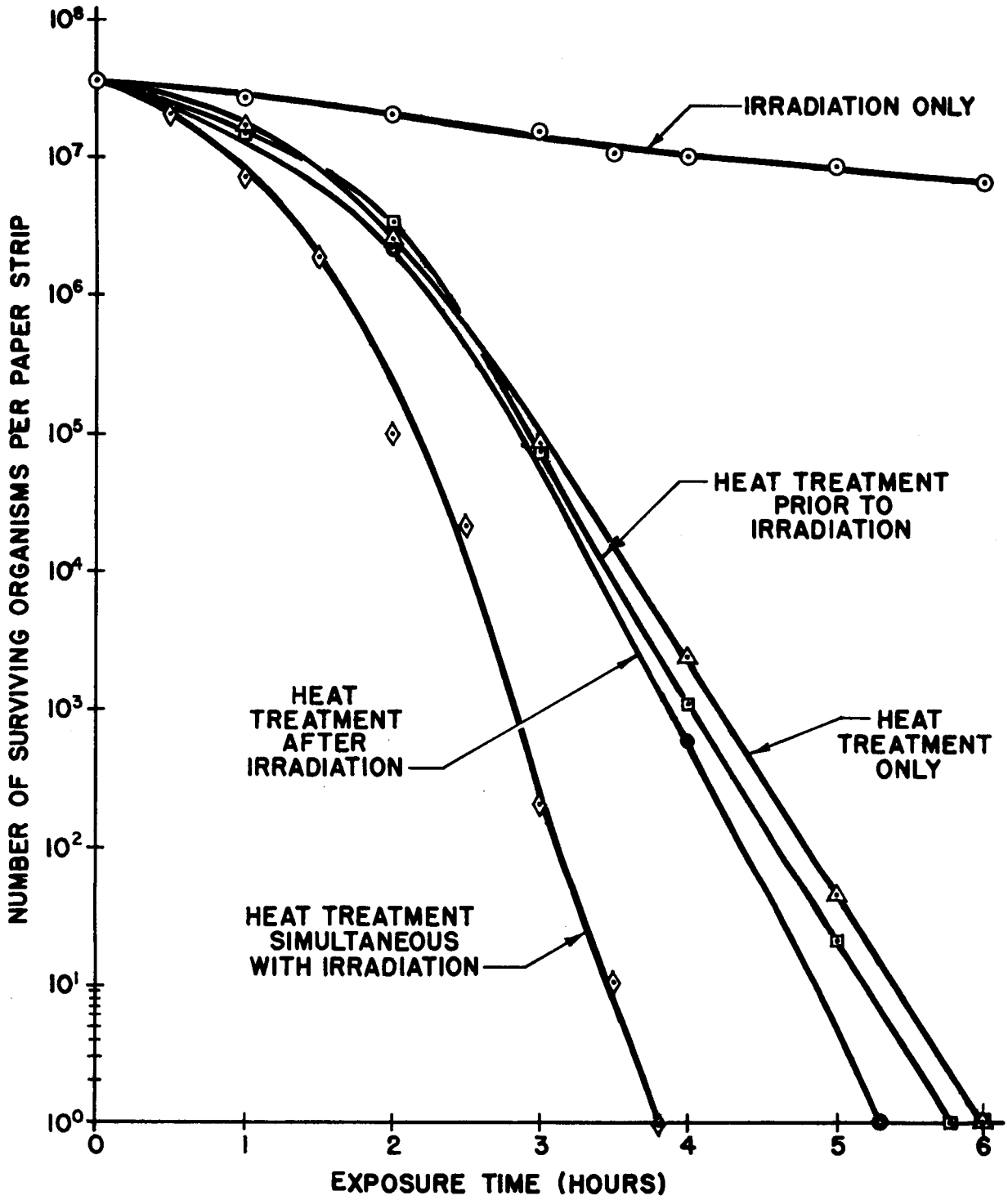

FIGURE 7. EFFECTS OF DRY HEAT TREATMENT AT 120°C. AND GAMMA IRRADIATION (2×10^4 RAD./HR.) FROM COBALT 60 ON DRY BACILLUS SUBTILIS VAR. NIGER SPORES ON PAPER STRIPS

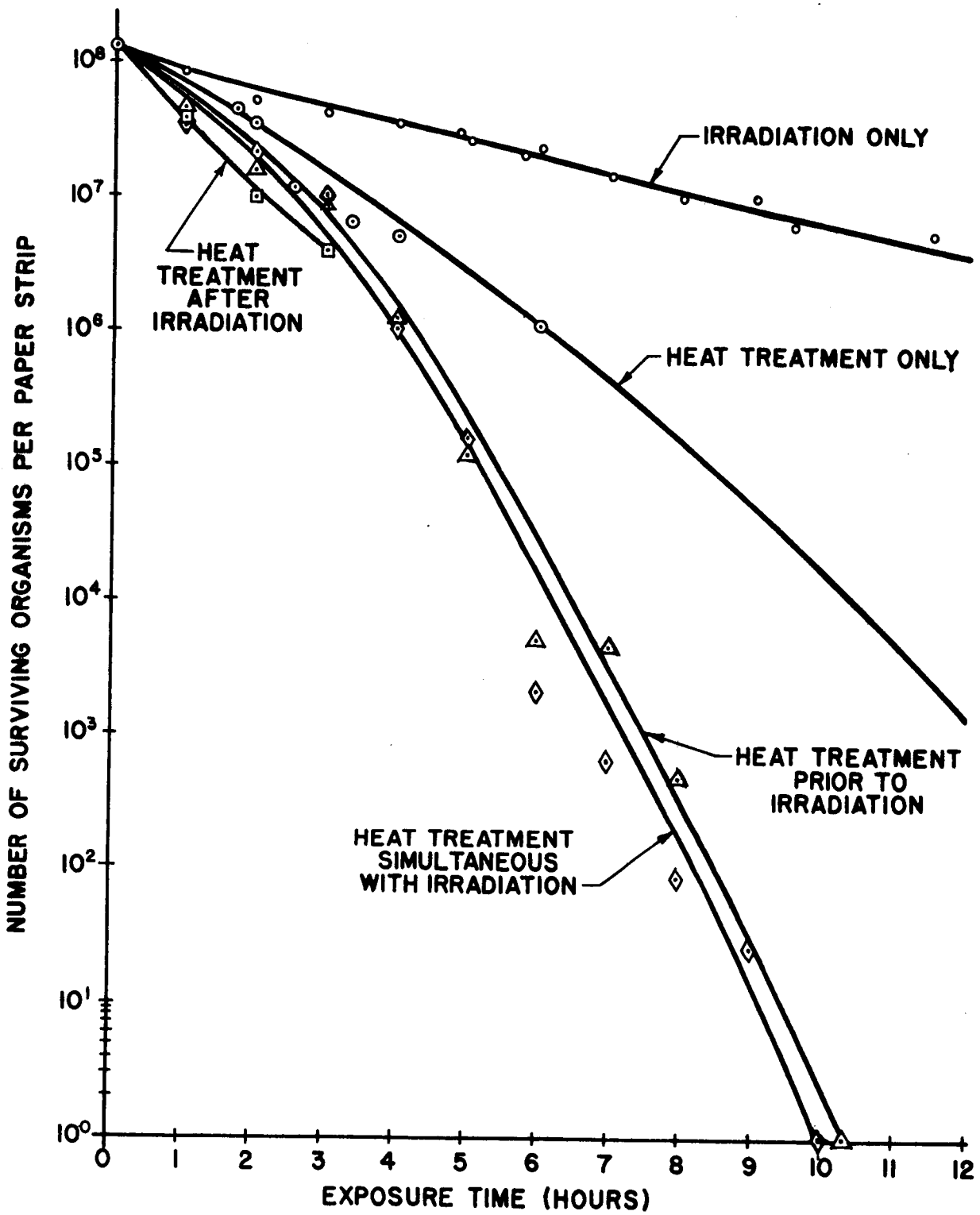

FIGURE 8. EFFECTS OF DRY HEAT TREATMENTS AT 120°C. AND GAMMA IRRADIATION (2×10^4 RAD./HR.) FROM COBALT 60 ON DRY BACILLUS COAGULANS SPORES ON PAPER STRIPS

TABLE I

Thermal death time values (in hours) for dry heat resistance of spores of various organisms in soil* in the temperature range 125-160°C in air.

Organism in soil FG	Spores per 0.1 g soil	Observation	Treatment Temperature 125	135	145	160°C
Bacillus subtilis var. niger	1.1×10^8	Survivors	8	6	1.5	0.7
		Sterile	16	8	2	0.9
Bacillus coagulans	2.2×10^7	Survivors	12	12	2	0.75
		Sterile		20		1
Soil isolate #69C**	4.3×10^7	Survivors	24	14	6	1
		Sterile	28	20	10	2
Soil isolate #541**	4.8×10^5	Survivors	80	36	16	4
		Sterile		40	20	6

* 0.1 g samples were employed for all tests.
** These organisms were not identified; both were gram positive spore-forming bacteria.

TABLE 2

Comparison of thermal death time values (in hours) for mesophilic aerobic microbial populations of FG soil[1] employing various recovery media in the temperature range 120-160°C in air.

Medium[2]	Observation	Treatment Temperature					
		120 (248)	125 (257)	135 (275)	140 (284)	145 (293)	160°C (320)°F
Thioglycollate broth	Survivors	55		8	6		1
	Sterile	60		16	8		3
Trypticase soy broth	Survivors	70			20		3
	Sterile	-					5
Tryptone glucose yeast extract broth	Survivors	320	220	100		45	10
	Sterile		320	110		55	15

1 0.1 g samples employed in all tests.
2 See text Appendix A.

TABLE 3

Thermal death time values (in days) for mesophilic aerobic microbial populations[1] of two soil samples at temperatures below 120°C in air

Sample	Observation	Temperature	
		80 (176)	100°C (212)°F
Soil FG	D value[2]	27	4.3
	F value[2]	188	30
Soil CO	D value	29	4.6
	F value	202	32

1 0.1 g samples were employed in all tests; aerobic mesophilic spore count was 1.5×10^6 per 0.1 g for soil FG and 1×10^5 per 0.1 g soil CO. Assays for sterility were made in thioglycollate broth.

2 D values were calculated from the equation of Stumbo(12). F values were calculated from the equation of Schmidt (11). The F values are for the destruction of one million (10^6) spores.

TABLE 4

Approximate thermal death times (in min.) for dry bacterial spores when treated in mineral oil versus treatment in air.

Organism	Treatment Temperature (°C)	Observation	Thermal Death Time		
			Strips Heated in air	Strips Heated in Mineral Oil	Strips added to Mineral Oil at Treatment temp.
Bacillus subtilis var. niger (Spore harvest D) 1×10^6 spores/strip	120	D value[1]	45	22	
		F value[2]	285	157	
	140	D value	6.6	4.8	
		F value	46	33	
	160	D value	1.1	2.4	0.6
		F value	7.8	17	4.2
Bacillus coagulans (Spore Harvest B) 1×10^6 spores/strip	120	D value	55.2	39	
		F value	386	272	
	140	D value	7.1	6.8	
		F value	50	48	
	160	D value	1.45	2.7	1
		F value	10	19	7

1 D values were calculated from the equation of Stumbo (12).

2 F values were calculated from the equation of Schmidt (11). These F values are for the destruction of one million (10^6) spores. Assays for sterility were made in trypticase soy broth.

XI ACKNOWLEDGEMENTS

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XIII PUBLICATIONS

The following oral presentation has been made on the work included in this report during 1962-1963.

Koesterer, M. G. and F. Smith* 1963 Investigation of the combined effects of gamma irradiation and dry heat on dry spores of Bacillus subtilis var. niger and B. coagulans.

Paper presented at the Central New York Branch, American Society for Microbiology, at Geneva, New York, November 23rd, 1963.

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XIV APPENDIX A

COMPOSITION OF CULTURE MEDIA

TRYPTONE GLUCOSE YEAST EXTRACT BROTH. (TGYE):
Made up with the following ingredients per liter:

Bacto-Tryptone (Difco[1])	5 g.
Bacto-Yeast Extract (Difco[1])	2.5 g.
Dextrose (glucose) (BBl[2], C. P. grade)	1 g.
Distilled water	1 liter

pH 7.0

SOIL EXTRACT BROTH:
Made up with the following ingr dients per liter:

Dextrose (glucose) (BBL[2], C. P. grade)	1 g.
K_2HPO_4	0.5 g.
Soil Extract*	100 ml.
Bacto-Agar (Difco)	15 g.
Tap water	900 ml.

pH 6.8-7.0

* Soil extract is prepared by treating equal amounts of soil and tap water in the autoclave for 30 min. A small amount of calcium carbonate is added and the soil suspension filtered through a double paper filter. The turbid filtrate is re-filtered until the extract comes through clear. The individual components of the medium can then be formulated as indicated above, bottled, and sterilized.

1 Difco Laboratories, Inc., Detroit, 1, Michigan
2 Baltimore Biological Laboratories, Inc., Baltimore 18, Maryland